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Conformational Changes of Transfer Ribonucleic Acid. The pH Phase Diagram under Acidic Conditions[†]

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ABSTRACT: We report measurements of the conformational behavior of tRNA^{Tyr} (*Escherichia coli*) as a function of hydrogen ion concentration below pH 7. Thermal melting curves at 150 mM Na$^+$, without Mg^{2+}, were measured at several wavelengths, and the rate of conformational change following a sudden pH increase from 4.4 to 7 was measured as a function of temperature. We also measured spectral changes accompanying titration at constant temperature. We conclude that tRNA^{Tyr} undergoes a conformational change centered around pH 5.5. The acid form contains some structural elements which must be dissociated before the native structure can be reformed at neutral pH, and it has greater proton affinity than the neutral form. It is only marginally less stable than the neutral form at pH 7 and therefore cannot be discounted as a possible functional intermediate. Addition of Mg^{2+} at acid pH alters the acid form but does not produce the native form.

Transfer RNA is frequently exposed to mildly acidic pH, one common reason being to stabilize the aminoacyl linkage against base-catalyzed hydrolysis. Little is known about the influence of pH on tRNA conformation, however. Our own interest in this problem began in the course of attempts to compare the melting of aminoacylated and uncharged tRNA, with the hope of detecting conformational or stability differences induced by aminoacylation. Retention of the aminoacyl linkage at elevated temperatures requires that one work at slightly acidic pH, and we soon discovered that there are dramatic differences between the thermal unfolding of tRNA in acid and neutral media.

In order to elucidate the effects of acid pH on tRNA conformation, we carried out a series of experiments closely analogous to those reported earlier (Cole *et al.*, 1972) concerning the influence of cation concentration on tRNA structure. The first step was to measure thermal denaturation profiles at varying pH. These revealed pH-dependent effects that indicated a conformational alteration of tRNA^{Tyr} around pH 5.5. We then performed pH-jump kinetic experiments in which the pH was changed from 4.4 to 7 by mixing, and the optical change followed as a function of time. The results indicate a slow conformational change with substantial activation energy, analogous to the effects observed on increasing salt concentration at neutral pH (Cole *et al.*, 1972). The result of these experiments is a phase diagram that shows the dependence of conformational state on pH at constant counterion concentration.

The experiments reported here refer to tRNA^{Tyr} from *Escherichia coli*, measured at 150 mM Na$^+$, generally in the absence of Mg^{2+}. We could not study systematically the effect of Mg^{2+} on thermal denaturation at acid pH, because acid catalysis of the destruction of 4-thiouridine (Lipsett, 1965) seems to be accelerated by Mg^{2+} (M. Bina-Stein, unpublished results), thus interfering with our spectral measurements at 335 nm. We did find, however, that tRNA exposed to acid pH in the presence of Mg^{2+} also showed a slow optical change on reneutralization, but the rate and activation energy for this reaction were affected by the presence of Mg^{2+}.

The reconversion of the acid conformation of tRNA^{Tyr} to the native state under charging conditions is sufficiently rapid that we could not determine whether the acid form can be aminoacylated or not. We will later report measurements

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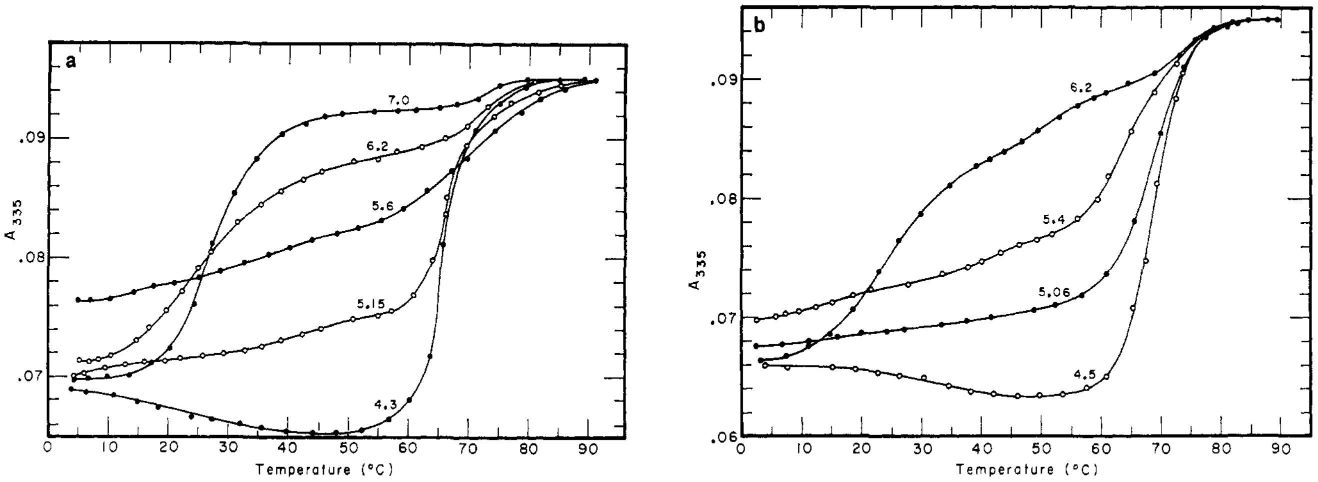

FIGURE 1: pH dependence of the melting behavior of tRNA^{Tyr} at 335 nm, in a buffer containing 150 mM Na$^+$: (a) tRNA$_{I}^{Tyr}$, (b) tRNA$_{II}^{Tyr}$. All melting curves were arbitrarily normalized by assuming the same absorbance at the highest temperature. pH values refer to measurements at room temperature.

(M. Bina-Stein and D. M. Crothers, in preparation) which indicate that for tRNA^{fMet} the acid form, which renatures more slowly than for tRNA^{Tyr}, cannot be aminoacylated. Furthermore, because of our better understanding of the details of thermal unfolding of tRNA^{fMet} (Crothers *et al.*, 1974), we are able to localize the acid-induced conformational change to the dihydrouridine helix and tertiary structure in that tRNA.

Materials and Methods

Buffers. Melting curves were measured at varying pH and constant Na$^+$ concentration. The acidic pH buffer contained 10 mM sodium acetate, 1 mM EDTA, and sufficient NaCl to bring the sodium ion concentration to 150 mM. The buffer used from neutral pH to pH 5.0 contained 10 mM sodium cacodylate instead of acetate. All pH measurements were performed with a Beckman pH meter at room temperature and at the T_m for a particular transition.

tRNA Samples. Unfractionated tRNA from *E. coli* K 12 was purchased from Schwarz. tRNA$_{I}^{Tyr}$ and tRNA$_{II}^{Tyr}$ were purified by the methods described by Armstrong *et al.* (1969) and Nishimura *et al.* (1967). The final preparations had tyrosine acceptor activity of 1450–1500 pmol/OD$_{260}$ for tRNA$_{I}^{Tyr}$ and 1650–1700 pmol/OD$_{260}$ for tRNA$_{II}^{Tyr}$.

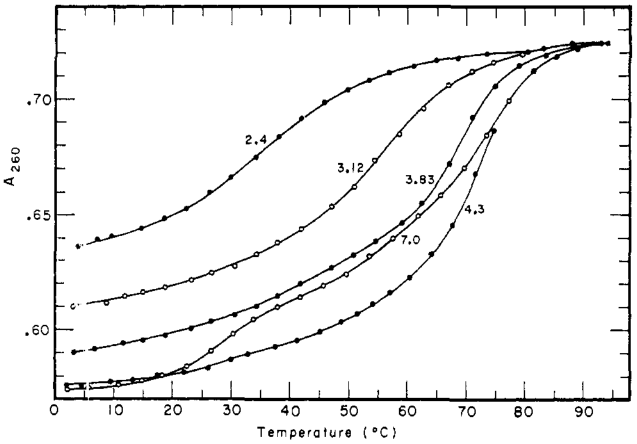

FIGURE 2: pH dependence of the melting behavior of tRNA$_{I}^{Tyr}$ at 260 nm; pH values refer to measurements at room temperature.

Acceptor activity assays were performed as described by Söll *et al.* (1967), using ^{14}C-labeled tyrosine (410 Ci/mol) purchased from New England Nuclear. A crude preparation of synthetase from *E. coli* Q13 was employed.

Melting Curves. Thermal transition curves were performed on a Cary Model 14 recording spectrophotometer as described previously (Cole *et al.*, 1972). tRNA samples were dialyzed extensively against 100 mM EDTA (pH 7) to chelate all Mg^{2+} present, followed by dialysis against the desired buffer. The transition midpoint T_m was taken as the temperature at which the absorbance had risen half-way between its initial and final values. Biphasic transitions were evaluated as described previously (Cole *et al.*, 1972). The temperature in the spectrophotometer cell was measured to an accuracy of 0.1° with a calibrated thermistor and appropriate resistance bridge.

pH-jump experiments were performed by raising the pH from 4.40 ± 0.01 to 7.0 ± 0.1 by adding an appropriate amount of a solution containing 10 mM sodium cacodylate and 140 mM NaOH. The mixing time was less than 20 sec. EDTA was omitted from the buffer in the pH-jump experiment. The absorbance change after mixing was followed as a function of time on a Cary Model 14 spectrophotometer.

Results

(*a*) *The Phase Diagram.* As we have previously reported, the thermal transition curves of tRNA species containing 4-thiouridine can be followed at 335 nm, as well as at wavelengths where the major bases absorb (Yang and Crothers, 1972; Cole *et al.*, 1972; Cole and Crothers, 1972). The 4-thiouridine absorbance is particularly sensitive to loss of tertiary structure and melting of the dihydrouridine helix (Cole *et al.*, 1972; Crothers *et al.*, 1974). The effect of pH on transition curves for tRNA^{Tyr} (150 mM Na$^+$, no Mg^{2+}) is shown in Figures 1 and 2. Referring to the curves for tRNA$_{I}^{Tyr}$ measured at 335 nm (Figure 1), the biphasic melting profile seen at neutral pH is strongly modified at lower pH. There is a progressive decrease in the amplitude of the first (low temperature) transition and a compensating increase in the amplitude of the second transition. At about pH 5 the first transition disappears completely. Lowering the pH from 5 to 4.1 produces no significant further change in either the 335- or 260-nm melting curve. At pH values below 4.1 the T_m of the second transition begins to decrease sharply with pH (Figure 2).

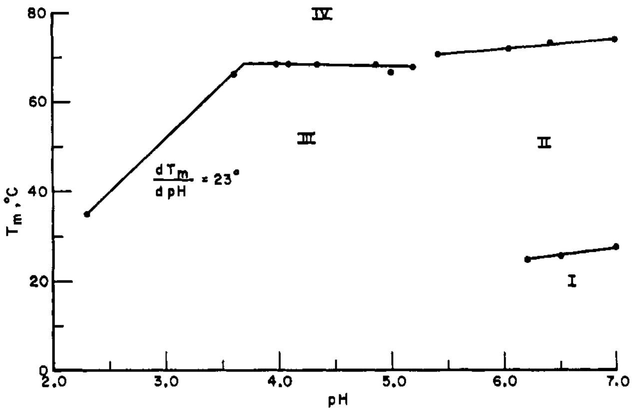

FIGURE 3: Variation of the transition temperatures for $tRNA_I^{Tyr}$ with pH, measured at T_m.

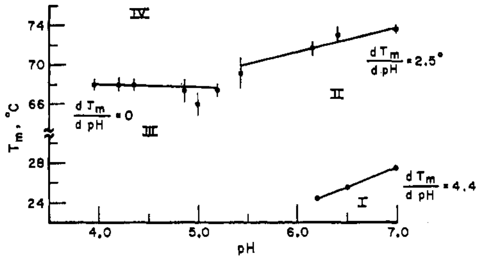

FIGURE 4: Variation of the transition temperatures for $tRNA_I^{Tyr}$ with pH on an expanded temperature scale. pH values were measured at T_m.

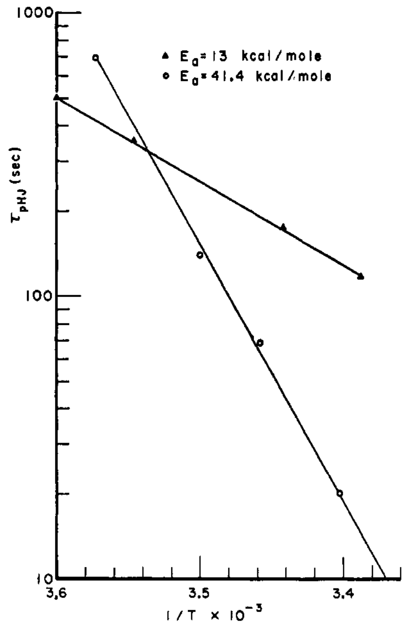

FIGURE 5: Temperature dependence of the pH-jump kinetics for $tRNA_I^{Tyr}$ in 150 mM Na^+. The conversion was from pH 4.4 to 7.0 in the absence (○) and presence (△) of 0.3 mM Mg^{2+}.

$tRNA_{II}^{Tyr}$ behaves similarly to $tRNA_I^{Tyr}$ (Figure 1) except for the difference in the melting behavior at neutral pH noted earlier (Yang and Crothers, 1972).

T_m values extracted from both the 335- and 260-nm transition curves are plotted against pH in Figure 3. The data between pH 4 and 7 are shown on an expanded temperature scale in Figure 4. The change in properties at about 5.5 is evident from two observations: the dependence of T_m on pH changes its slope around that point, and the amplitude of the first transition vanishes below pH 5.5. It therefore seems probable that the structure that melts in two steps at neutral pH is replaced by a new form. This implies a phase diagram with IV zones, as shown in Figure 3. I and II are the forms found at neutral pH and "high salt" (Cole *et al.*, 1972); these were interpreted as the native structure (I) and a cloverleaf lacking dihydrouridine helix and tertiary structure (II). IV is the single strand, and III is the new "acid form" of $tRNA^{Tyr}$. As will be seen below, the degree of protonation of both III and IV changes with pH, but there is no evidence for a further distinct region of the conformational phase diagram.

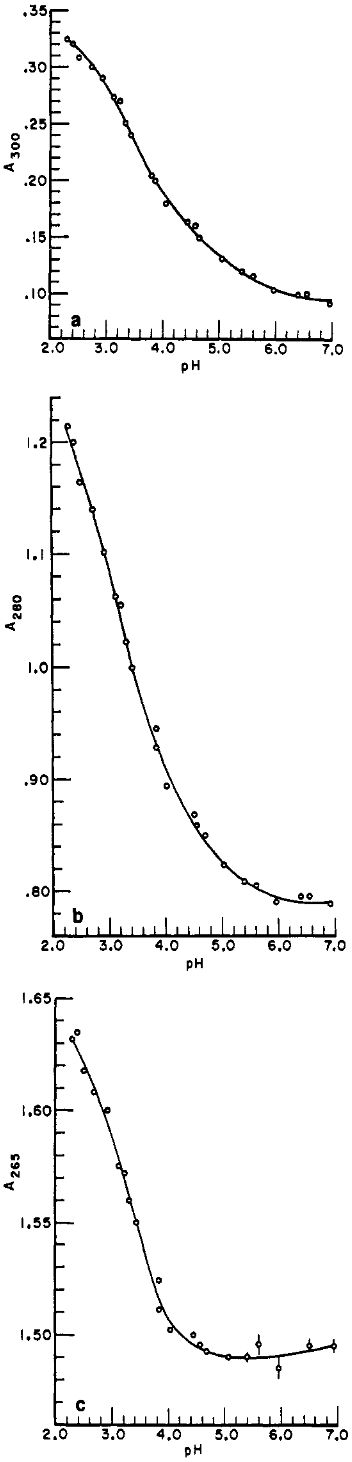

FIGURE 6: Spectrophotometric titration of $tRNA_I^{Tyr}$ at 20° at various wavelengths.

(*b*) *pH-Jump Experiments.* In our earlier work on the variation of tRNA conformation with Na^+ concentration, we

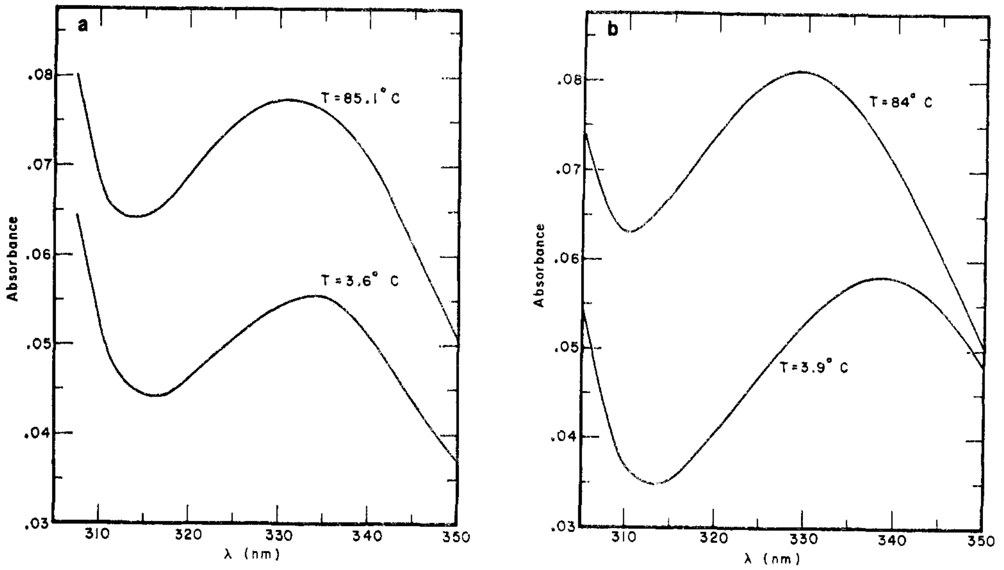

FIGURE 7: Absorbance spectra of tRNA$_I^{Tyr}$ in 150 mM Na$^+$ at (a) pH 4.3 and (b) pH 7.0.

showed that conversion of tRNA between two ordered phases occurs slowly and with appreciable (25–61 kcal/mol) activation energy (Cole *et al.*, 1972). We found the same to be true for conversion of form III to I in the acid phase diagram. Addition of alkali to raise the pH from 4.4 to 7 yielded an optical change sufficiently slow that it could be investigated by simple mixing methods. The data were adequately fit by a single exponential after the 20 sec deadtime for mixing. Figure 5 shows the exponential decay time τ, measured at 260 nm, as a function of reciprocal temperature. The activation energy obtained from the Arrhenius equation is 41 ± 2 kcal/mol.

When Mg^{2+} was present in the buffer before the pH jump, the kinetic behavior was considerably modified. Two decay components were observed in the time after completion of mixing; data points for the slower exponential are included in Figure 5. Since the rate and activation energy (14 kcal/mol) are very different from those observed without Mg^{2+}, it seems likely that a further modification of the acid form is induced by Mg^{2+}.

(*c*) *Optical Titration of tRNA$_I^{Tyr}$*. The absorbance increase upon addition of acid to tRNA$_I^{Tyr}$ at 20° is shown at several wavelengths in Figure 6. Comparison with the melting curves (Figures 1 and 2) and the phase diagram (Figure 3) shows that pH-dependent spectral changes are substantial under conditions where melting is not yet appreciable, for example in the pH range from 4.5 to 5.5. These premelting changes can be seen at 280 and 300 nm, but not at 265 nm. This is the result expected for protonation of cytosine, which has been held responsible for the premelting protonation of DNA and the attendant spectral changes (Zimmer and Venner, 1966; Zimmer *et al.*, 1968).

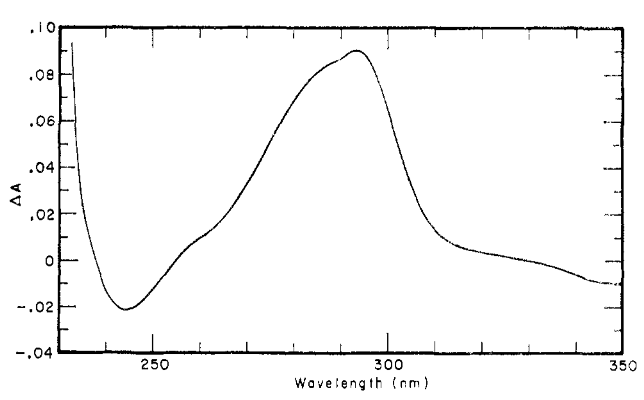

FIGURE 8: Difference spectra for a change in the pH of a solution of tRNA$_I^{Tyr}$ in 150 mM Na$^+$. A base line was first obtained by measuring the absorbance spectrum with identical tRNA^{Tyr} solutions at pH 4.3 in both sample and reference beams. The pH of the reference compartment was raised to 7.0 as described under methods. An equal amount of buffer was added to the sample compartment. The concentration of tRNA^{Tyr} before the addition of buffer was 1.54 A_{260}/ml. $\Delta A = A_{\text{pH } 4.3} - A_{\text{pH } 7.0}$.

The large absorbance increase that accompanies melting begins below pH 4.5, as seen from the titration curve measured at 265 nm. Acid denaturation is evidently much less cooperative than for DNA, since the absorbance changes for tRNA cover a much wider pH range than is found for DNA (Bunville *et al.*, 1965; Zimmer *et al.*, 1968). Precipitation of tRNA^{Tyr} at pH 2.3 prevents observations at lower pH values.

(*d*) *Spectral Changes.* The effect of acidification on the absorbance spectrum of tRNA^{Tyr} is summarized in Figures 7 and 8. Acidification (pH 4.5, no Mg^{2+}) shifts the low temperature 4-thiouridine absorbance maximum from 337 nm (pH 7) to 334 nm. Upon heating the maximum is near 330 nm in both cases. The difference spectrum found on acidification at low temperature is shown in Figure 8. There is little change around 260 nm, and the position of the absorbance maximum there is unaffected. There is a maximum in the difference spectrum near 293 nm, again consistent with assignment of the principal effect to protonation of cytosine.

Discussion

The results reported here indicate that one cannot assume that tRNA has the same conformation at pH values below 6 as it has at neutral pH. Two observations support the existence of a conformational alteration near pH 5.5. First, the thermal transition behavior changes character there, including disappearance of the biphasic melting curve and a change in the slope of the dependence of T_m on pH. Second, a slow conversion between the two forms, with 41 kcal/mol activation energy for conversion of the acid structure to the native form, can be observed optically when the pH is changed rapidly. These findings support previous indications of an acid-induced change in tRNA structure, both for tRNA^{Phe} (Wong *et al.*, 1973) and for the precursor of tRNA^{Tyr} (Altman and Stark, 1974).

The finding of an appreciable activation energy for forming the neutral pH structure from the acid form implies that the acid form contains some "incorrect" structure. This conclusion rests on the following logic: simple reactions in which double helix (Craig *et al.*, 1971; Pörschke and Eigen, 1971) or tertiary structure (Cole and Crothers, 1972; Crothers *et al.*, 1974) are formed from unbonded bases are fast (less than 100 msec) and have negligible activation energy. When the reaction is slow and has a large activation energy, its rate is limited by the necessity for dissociation of the "incorrect" structure before the native structure can be formed (Yang and Crothers, 1972). The activation energy arises from the enthalpy of melting the "incorrect" structure.

The reason the "incorrect" structure is formed at acid pH is that protonation provides an alternative structure more stable than the native form. The diagram in Figure 4 shows that the acid form binds protons better than the native form. According to simple theory (Crothers, 1971) the variation of T_m with activity a of a bound ligand (proton) is $dT_m/d \ln a = [(r_h - r_c)/\Delta H]RT_m^2$, where ΔH is the enthalpy of helix formation, r_h the number of protons bound to the ordered form, and r_c the protons bound to the coil. According to Figure 4, $(r_h - r_c)$ is zero for the acid form between pH 4 and 5, while $(r_h - r_c)$ is negative for the neutral form, between pH 5.5 and 7. Since both forms melt to the same final coil state, the proton binding by the acid form exceeds that by the native form at a particular pH. It is evident that the acid form has a special affinity for protons, but the site of protonation cannot be specified at present.

It should also be noted that the acid and native forms do not differ greatly in stability at neutral pH. Thus the extrapolated T_m of the acid form is only about 7° below that of the native form at pH 7. Furthermore, even at pH 6.2 there are appreciable changes in the melting curve resulting from contributions by the acid structure (Figure 1). Hence the acid form cannot be ruled out as a possible functional intermediate at pH 7.

Our present experiments do not provide sufficient basis for specifying the "incorrect" structure in tRNA^{Tyr} at pH below 5.5. By analogy with $\text{tRNA}^{\text{fMet}}$ (M. Bina-Stein and D. M. Crothers, in preparation) it is reasonable to suppose that the alterations involve tertiary structure and the dihydrouridine helix. This is consistent with disappearance of the first melting transition, which reflects melting of tertiary structure and probably also of the dihydrouridine helix, when the pH is below 5.5. The detailed nature of the structural change remains to be elucidated, however.

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